$CH_3Hg^{II}$, 5.9 mM BSA, and 18.5 mM cysteine, it was estimated by exclusion chromatography that 46% of the BSA was complexed. From mass-balance relationships and the $K_{fc}$ for the $CH_3Hg^{II}$–cysteine complex, log $K_{fc}$ for the $CH_3Hg^{II}$–BSA complex is calculated to be 11.0. This value and the $K_{fc}$ for the hemoglobin complex are both smaller than the conditional formation constants for $CH_3Hg^{II}$ complexes with a variety of monothiols (ranging from 11.1 to 11.7). Because of the dependence of $K_{fc}$ on the acid–base properties of the sulfhydryl groups, which have not been quantitatively characterized for hemoglobin or BSA, it is not possible to draw any conclusions regarding the intrinsic strength of the $CH_3Hg^{II}$–protein binding.

For purposes of elucidating the chemistry of $CH_3Hg^{II}$ toxicology, however, the conditional formation constants at pH 7.4 are the quantities of interest; the relative magnitudes of the $K_{fc}$ values indicate the relative affinities of the ligands for $CH_3Hg^{II}$. The conditional formation constants combined with the normal levels of GSH, hemoglobin, and ergothioneine in human erythrocytes[21] lead to the prediction that of the $CH_3Hg^{II}$ in human erythrocytes, 59% will be complexed by GSH, 41% by hemoglobin, and 0.001% by ergothioneine. This prediction is in agreement with results from $^1H$ NMR measurements[6] on intact human erythrocytes containing $CH_3Hg^{II}$ and gel-filtration studies of $CH_3Hg^{II}$ in hemolyzed erythrocytes.[22]

It is of interest to also compare these conditional formation constants to those of complexes with other biological molecules containing sulfhydryl groups and with sulfhydryl molecules used as antidotes for $CH_3Hg^{II}$ poisoning.[8] Log $K_{fc}$ values at pH 7.4 for $CH_3Hg^{II}$ complexes with other biological molecules are cysteine (11.57) and homocysteine (11.15) and with antidote molecules are mercaptosuccinic acid (11.68), penicillamine (11.33), and *N*-acetylpenicillamine (11.20). $K_{fc}$ for the hemoglobin complex is somewhat less than those of the complexes with the other biological thiols and the antidote molecules. $K_{fc}$ for the GSH complex is similar to that of the cysteine complex; however, GSH is much more abundant than free cysteine in cellular systems.[4] $K_{fc}$ for the mercaptosuccinic acid complex is larger than that for the GSH complex, which correlates with the greater effectiveness of mercaptosuccinic acid as compared to that of penicillamine as an antidote for $CH_3Hg^{II}$ poisoning in animal studies.[23]

The method used to measure $K_{fc}$ for the $CH_3Hg^{II}$ complex of hemoglobin is direct, precise, and should be applicable to the measurement of other metal–protein binding constants. The basis of the method is the measurement of the extent of complexation of a small ligand in the presence of the macromolecule. The key is the selective observation of resonances from the small ligand, which can be done either with the method used here[9] or with the spin–echo Fourier transform method.[11] If exchange of the small ligand between its free and complexed forms is fast on the NMR time scale, the extent of complexation of the small ligand can be obtained directly from the chemical shifts of exchange-averaged resonances. If exchange is slow, the extent of complexation can be obtained from the relative intensities of the free and complexed resonances. We are investigating the application of this method to other metal–protein systems.

**Acknowledgment.** This research was supported by the National Sciences and Engineering Research Council of Canada through their Strategic Grants program and by The University of Alberta. Financial support of R.S.R. by an I. W. Killam Scholarship is gratefully acknowledged.

**Registry No.** GSH, 70-18-8; ergothioneine, 497-30-3.

(20) Berg, G. C.; Miles, E. F. *Membr. Biochem.* **1978**, *2*, 117.
(21) Glutathione (2.2 mM), hemoglobin (5.2 mM), and ergothioneine (0.18 mM): Natelson, S.; Natelson, E. A. "Principles of Applied Clinical Chemistry": Plenum Press: New York, 1978; Vol. 2.
(22) Naganuma, A.; Imura, N. *Toxicol. Appl. Pharmacol.* **1979**, *47*, 613.
(23) Aaseth, J.; Friedheim, E. A. *Acta Pharmacol. Toxicol.* **1978**, *42*, 248.

# Kinetics of the Protonation of Buffer and Hydration of $CO_2$ Catalyzed by Human Carbonic Anhydrase II

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**Abstract:** The interaction of external buffer with human carbonic anhydrase II in the catalyzed hydration of $CO_2$ has been examined for several cationic and zwitterionic buffers over the pH range 6.8–9.0. The rate of change of absorbance of a pH indicator, measured by stopped-flow spectrophotometry, was used to determine initial velocities. We found that the rate-limiting proton transfer between human carbonic anhydrase II and external buffer is dependent on the p$K$ difference between donor and acceptor species in a manner consistent with proton transfer between small molecules. The rate constant for the proton transfer follows a Brønsted curve that reaches a plateau at a value of $1 \times 10^9$ $M^{-1}$ $s^{-1}$, very close to that found for proton transfer between small molecules. The transition region of the Brønsted plot indicates that the donor group on the enzyme has a $pK_a = 7.6 \pm 0.6$, which is consistent with proton transfer directly between the active site and buffer or between a proton shuttle group on the enzyme of $pK_a$ near 7 and buffer.

Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reaction shown in (1), the hydration of $CO_2$ to produce bicarbonate and a proton.[1] Under physiological conditions the

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

turnover number for $CO_2$ hydration catalyzed by human carbonic anhydrase II (the high activity isozyme) is large, near $10^6$ $s^{-1}$. Since the catalyzed reaction produces a proton, it is necessary that a proton be transported out of the active site at a rate at least as rapid as the hydration rate. The rapid rate of proton transfer from the enzyme to solvent cannot be accounted for by proton transfer to water or $OH^-$ at pH near 7, water because it is a poor proton acceptor and $OH^-$ because it is not present at sufficient concentration. It has been pointed out that removal of this proton from the enzyme may be accomplished mainly by buffers in

(1) Pocker, Y.; Sarkanen, S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1978**, *47*, 149–274.

0002-7863/82/1504-6737$01.25/0 

solution acting as proton acceptors.[2-4] This proposal was subsequently verified by experiments that showed a decrease in catalyzed, initial velocity of hydration[5] and a decrease in catalyzed exchange of $^{18}O$ between $CO_2$ and $H_2O$[6] as buffer concentration was decreased below 10 mM. Equation 2 shows the catalytic

$$E + CO_2 + H_2O \overset{k_1}{\rightleftharpoons} E_{H_2O}{}^{CO_2} \overset{k_2}{\rightleftharpoons} E_{HCO_3^-}{}^{H^+} \overset{k_3}{\rightleftharpoons} EH^+ + HCO_3^- \quad (2)$$

interconversion of $CO_2$ and $HCO_3^-$. Here, $EH^+$ is the form of the enzyme with the activity-controlling group protonated, and E is the unprotonated form. A wide body of evidence indicates that the activity-controlling group is a zinc-bound water molecule that dissociates to zinc-bound OH with a $pK_a$ of about 7 at physiological conditions of ionic strength. Equation 3 is the simplest scheme that describes the interaction of external buffer (B and $BH^+$) with the enzyme.

$$EH^+ + B \overset{k_4}{\rightleftharpoons} E + BH^+ \quad (3)$$

From (2) and (3) it is evident that the interaction of buffer with the enzyme plays an important role in the overall catalytic cycle. We have examined this interaction by measuring the initial velocity of the catalyzed hydration reaction in the presence of various buffers by stopped-flow spectrophotometry in which the rate of change of a pH indicator is observed. We have found that, at least for low concentrations of buffer (<12 mM), the rate-limiting proton transfer between human carbonic anhydrase II and external buffer is dependent on the $pK$ difference between the donor and the acceptor species in a manner consistent with proton transfer between small molecules. That is, the rate constant for proton transfer follows a Brønsted curve that reaches a limiting maximum value of $1 \times 10^9$ $M^{-1}$ $s^{-1}$, very close to the value found for proton transfer between small molecules. The transition region of the Brønsted curve indicates that the donor group on the enzyme has a $pK_a$ of approximately 7.5, which is consistent with proton transfer directly between the active site and buffer or indirectly with the participation of a proton shuttle group on the enzyme with a similar $pK_a$ value.

## Experimental Section

**Materials.** Carbonic anhydrase II was obtained from human erythrocytes of outdated blood by an affinity chromatography procedure.[7] Enzyme concentrations were estimated from absorbance measurements at 280 nm by using a molar extinction coefficient of $5.3 \times 10^4$ $M^{-1}$ $cm^{-1}$.[8] Phenol red, chlorophenol red, *p*-nitrophenol, *m*-cresol purple, thymol blue, Hepes,[9] triethanolamine hydrochloride, 1-methylimidazole, 1,2-dimethylimidazole, Ches,[9] and Mes[9] were obtained from Sigma. Bromocresol purple, 3,5-lutidine, 3,4-lutidine, 2,4-lutidine, 4-methylimidazole, and Ted[9] were obtained from Aldrich. Ted, 1,2-dimethylimidazole, and all of the lutidines were purified by distillation under reduced pressure. Distilled, deionized water was passed through a ion-exchange column (Cole-Parmer 1506-35) prior to use to prevent contamination with adventitious metal ions. All other reagents were used without further purification.

**Kinetic Methods.** Saturated solutions of $CO_2$ were prepared by bubbling $CO_2$ gas into water in a vessel maintained at 25.0 °C, and dilutions were prepared in the absence of air by coupling two syringes as described by Khalifah.[10] Carbon dioxide concentrations were calculated on the basis of a concentration of 33.8 mM at 25 °C for a saturated solution.[11]

Initial rate measurements of $CO_2$ hydration were carried out on a Durrum-Gibson stopped-flow spectrophotometer equipped with a Nicolet Explorer Model 206 digital oscilloscope. All experiments were performed at 25 °C with the ionic strength maintained at 0.20 by adding the appropriate amount of $Na_2SO_4$. One drive syringe contained $CO_2$, and the second contained enzyme, buffer, and indicator. For experiments using bicarbonate as inhibitor, the bicarbonate was added to the contents of the second syringe; these experiments were performed at pH near 8, and the addition of bicarbonate did not significantly change the pH of the buffer. The initial velocity of hydration ($v$) was determined according to (4), with $dA/dt$, the rate of change of absorbance of indicator, de-

$$v = -\left(\frac{d[CO_2]}{dt}\right)_{initial} = Q_0\left(\frac{dA}{dt}\right)_{initial} \quad (4)$$

termined by least-squares analyses of the average of four replicate absorbance *vs.* time traces, each comprising less than 10% of the complete reaction. Initial velocities were corrected for the uncatalyzed rate where necessary. This analysis was facilitated by interfacing the oscilloscope with a Hewlett-Packard 9835B calculator. The buffer factor ($Q_0$) relates changes in absorbance of indicator to changes in the concentration of $H^+$ and was calculated for each buffer–indicator pair as described by Khalifah.[10] The $pK_a$ values for buffers and indicators were determined by direct and spectrophotometric titration, respectively, at 25 °C at an ionic strength of 0.20 achieved by adding the necessary amount of $Na_2SO_4$. Using these same conditions, we also measured $\Delta\epsilon$, the difference in the molar extinction coefficient of the acidic and basic forms of the inidicator, determined at the wavelength used in the stopped-flow experiments. The buffer–indicator pairs used in this study, their $pK_a$ values, wavelengths used, and $\Delta\epsilon$ values were as follows: Mes ($pK_a$ = 6.1) and 3,5-lutidine ($pK_a$ = 6.2) with chlorophenol red ($pK_a$ = 6.3, $\lambda$ = 574 nm, $\Delta\epsilon = 1.77 \times 10^4$ $M^{-1}$ $cm^{-1}$); 3,4-lutidine ($pK_a$ = 6.6) and 2,4-lutidine ($pK_a$ = 6.8) with bromocresol purple ($pK_a$ = 6.8, $\lambda$ = 588 nm, $\Delta\epsilon = 3.00 \times 10^4$ $M^{-1}$ $cm^{-1}$); 1-methylimidazole ($pK_a$ = 7.2) with *p*-nitrophenol ($pK_a$ = 7.1, $\lambda$ = 400 nm, $\Delta\epsilon = 1.83 \times 10^4$ $M^{-1}$ $cm^{-1}$); Hepes ($pK_a$ = 7.5), triethanolamine ($pK_a$ = 7.8), and 4-methyimidazole ($pK_a$ = 7.8) with phenol red ($pK_a$ = 7.5, $\lambda$ = 557 nm, $\Delta\epsilon = 5.59 \times 10^4$ $M^{-1}$ $cm^{-1}$); 1,2-dimethylimidazole ($pK_a$ = 8.2) with *m*-cresol purple ($pK_a$ = 8.3, $\lambda$ = 578 nm, $\Delta\epsilon = 3.81 \times 10^4$ $M^{-1}$ $cm^{-1}$); Ted ($pK_a$ = 9.0) and Ches ($pK_a$ = 9.2) with thymol blue ($pK_a$ = 8.9, $\lambda$ = 590 nm, $\Delta\epsilon = 2.43 \times 10^4$ $M^{-1}$ $cm^{-1}$). The enzyme concentration in kinetic experiments was typically 69 nM.

**Analysis of Kinetic Data.** Estimation of kinetic constants from initial rates vs. substrate concentrations was accomplished by using a weighted, linear least-squares method,[12] with $v^4$ weights, where $v$ is the observed initial rate in (4). Analysis of data illustrated in Figure 3 was done similarly, but with the more appropriate $(k_4)^2$ weights[13] (see Discussion).

Table I. Steady-State Constants for the Hydration of $CO_2$ and Protonation of Buffer Catalyzed by Human Carbonic Anhydrase II[a]

| | $pK_a$ | pH | $K_m^B$,[b] mM | $k_4$,[b] $M^{-1}$ $s^{-1}$ |
|---|---|---|---|---|
| Mes | 6.1 | 6.8 | 3.2 | $3.1 \times 10^7$ |
| 3,5-lutidine | 6.2 | 6.9 | 3.3 | $6.2 \times 10^7$ |
| 3,4-lutidine | 6.6 | 7.3 | 2.7 | $6.6 \times 10^7$ |
| 2,4-lutidine | 6.8 | 7.5 | 2.5 | $7.4 \times 10^7$ |
| 1-methylimidazole | 7.2 | 8.0 | 2.1 | $2.9 \times 10^8$ |
| Hepes | 7.5 | 8.2 | 3.5 | $2.1 \times 10^8$ |
| triethanolamine | 7.8 | 8.4 | 1.2 | $5.4 \times 10^8$ |
| 4-methylimidazole | 7.8 | 8.4 | 1.3 | $1.2 \times 10^9$ |
| 1,2-dimethylimidazole | 8.2 | 8.5 | 1.3 | $4.6 \times 10^8$ |
| Ted | 9.0 | 8.9 | 1.1 | $7.9 \times 10^8$ |
| Ches | 9.2 | 9.0 | 0.5 | $1.5 \times 10^9$ |

[a] Human carbonic anhydrase II was present at 69 nM, 25 °C, with ionic strength maintained at 0.2 with $Na_2SO_4$. [b] $K_m^B$ is the Michaelis constant for buffer corrected to represent the basic form of buffer at the values of pH indicated. The Michaelis constant for $CO_2$ was determined to be 8 ± 2 mM. The maximum value of $k_{cat}$ is near $8 \times 10^5$ $s^{-1}$. In the catalytic scheme considered here, $k_{cat}/K_m^B = k_4$.

(2) Khalifah, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 1986–1989.
(3) Prince, R. H.; Woolley, P. R. *Bioorg. Chem.* **1973**, *2*, 337–344.
(4) Lindskog, S.; Coleman, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2505–2508.
(5) Jonsson, B.-H.; Steiner, H.; Lindskog, S. *FEBS Lett.* **1976**, *64*, 310–314.
(6) Silverman, D. N.; Tu, C. K. *J. Am. Chem. Soc.* **1975**, *97*, 2263–2269.
(7) Khalifah, R. G.; Strader, D. J.; Bryant, S. H.; Gibson, S. M. *Biochemistry* **1977**, *16*, 2241–2247.
(8) Edsall, J. T.; Mehta, S.; Myers, D. V.; Armstrong, J. M. *Biochem. Z.* **1966**, *345*, 9–36.
(9) Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Ted, 1,4-diazabicyclo-[2.2.2]octane; Ches, 2-(cyclohexylamino)ethanesulfonic acid.
(10) Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573.
(11) Pocker, Y.; Bjorkquist, D. W. *J. Am. Chem. Soc.* **1977**, *99*, 6537–6543.
(12) Wilkinson, G. N. *Biochem. J.* **1961**, *80*, 324–332. Cleland, W. W. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1967**, *29*, 1–32.
(13) Reich, J. G. *FEBS Lett.* **1970**, *9*, 245–251.

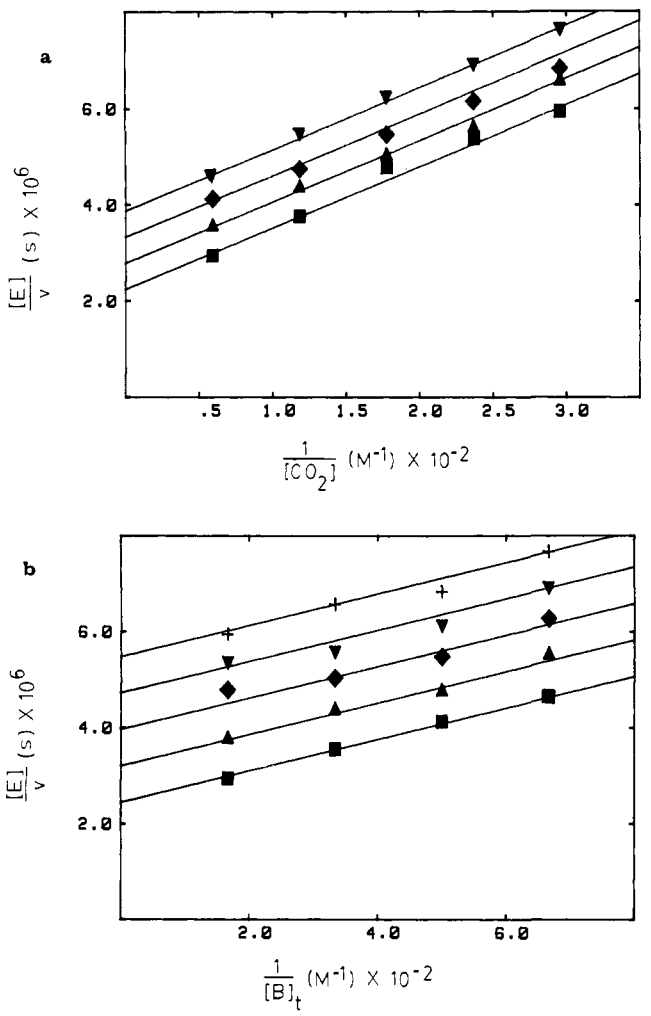


**Figure 1.** Dependence of $[E]/v$ on $1/[CO_2]$ and $1/[B]_t$ for the hydration of $CO_2$ and protonation of buffer catalyzed by human carbonic anhydrase II present at 69 nM. Ionic strength was maintained at 0.20 with $Na_2SO_4$, temperature was 25 °C and pH 8.5. (a) [1,2-dimethylimidazole] = 6.0 mM (■), 3.0 mM (▲), 2.0 mM (♦), 1.5 mM (▼) (these values are total concentration of buffer). (b) Replot of same data for $[B]_t$ = 1,2-dimethylimidazole, $[CO_2]$ = 17 mM (■), 8.5 mM (▲), 5.6 mM (♦), 4.2 mM (▼), 3.4 mM (+). Lines drawn through data points were determined by weighted least-squares analysis (see text) and calculated for $k_{cat} = 5.9 \times 10^5\ s^{-1}$, $k_m^{CO_2} = 7.6$ mM, $K_m^B = 2.0$ mM.

## Results

The dependence of initial velocity upon concentration of $CO_2$ and external buffer was examined over the pH range 6.8 to 9.0 for a variety of cationic and zwitterionic buffers listed in Table I with values of $pK_a$ from 6.1 to 9.2. Where feasibile, the pH of each analysis was chosen such that the concentration of the basic form of the buffer was much greater than the protonated form. For three buffers (1,2,-dimethylimidazole, Ted, Ches) this condition could not be maintained due to rapid uncatalyzed hydration at pH near and above 9. However, linear plots (such as in Figure 1) were still obtained for these buffers, a result which indicates that the back-reaction involving protonated buffer with enzyme is relatively unimportant under our conditions. To verify this, we performed experiments using the buffers 3,5-lutidine, 2,4-lutidine, and 1-methylimidazole at a pH equal to the $pK_a$ of the buffer. In each case, kinetic parameters identical with those in Table I were obtained. In addition, concentration of buffer was limited to 12 mM to lessen the possibility of the binding of buffer to multiple (and possibly inhibitory) sites. The data in Figure 1a are typical for the dependence of initial velocity upon the concentration of $CO_2$ as substrate in the presence of each of the buffers listed in Table I. The parallel lines in this plot demonstrate the independence of $k_{cat}/K_m^{CO_2}$ (the reciprocal of the slope in Figure 1a) toward concentration of buffer. Figure 1b is a replot of Figure 1a with the concentration of buffer varied at constant $CO_2$.

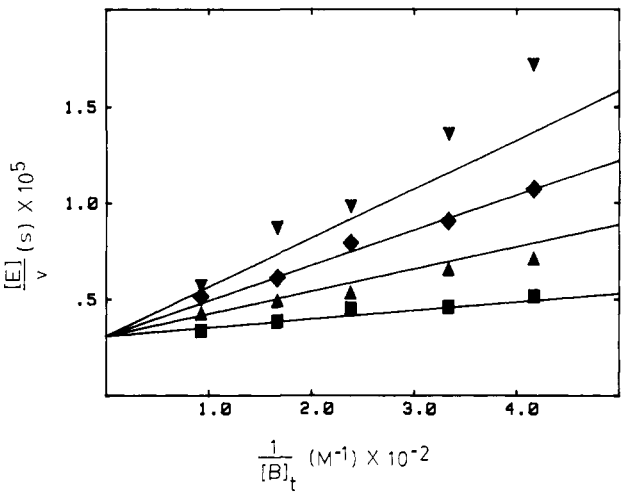


**Figure 2.** Inhibition by $HCO_3^-$ of the catalyzed hydration of $CO_2$. Human carbonic anhydrase II was present at 69 nM, 25.0 °C, ionic strength = 0.20 (maintained with $Na_2SO_4$), $[CO_2]$ = 8.5 mM, $[B]_t$ = total triethanolamine, pH 8.4, $[HCO_3^-]$ = 0 mM (■), 17 mM (▲), 33 mM (♦), 50 mM (▼). Lines drawn through data points were determined by weighted least-squares analysis (see text) and calculated for the following apparent values at constant and unsaturating $[CO_2]$: $k_{cat} = 3.2 \times 10^5\ s^{-1}$, $K_m^B$ = 1.4 mM (based on total buffer), $K_i^{HCO_3^-}$ = 10.5 mM.

Table II. Apparent Inhibition Constant $K_i^{HCO_3^-}{}_{app}$ for the Inhibition by $HCO_3^-$ of the Hydration of $CO_2$ Catalyzed by Human Carbonic Anhydrase II in the Presence of Buffer[a]

| buffer | pH | $K_i^{HCO_3^-}{}_{app}$, mM |
|---|---|---|
| triethanolamine | 8.4 | 10 ± 1 |
| Hepes | 8.2 | 10 ± 1 |
| 4-methylimidazole | 8.4 | 5.4 ± 0.3 |
| 1-methylimidazole | 8.0 | 6.5 ± 0.4 |

[a] Human carbonic anhydrase II was present at 69 nM, ionic strength 0.20 (maintained with $Na_2SO_4$), and 25 °C. Linear competitive inhibition by $HCO_3^-$ was observed in all cases. The apparent inhibition constant is related to the true substrate dissociation constant as indicated by (7), assuming (2) and (3) for the catalysis.

The rate equation for the catalyzed hydration of $CO_2$ according to (2) and (3) can be written in the form of (5), in which $k_{cat}$ and $K_m^{CO_2}$ are the turnover number and Michaelis constant for hydration in the presence of excess buffer and $k_4$ is the bimolecular rate constant for the transfer of a proton from enzyme to buffer.

$$\frac{[E]_t}{v} = \frac{1}{k_4[B]} + \frac{1}{k_{cat}}\left(1 + \frac{K_m^{CO_2}}{[CO_2]}\right) \tag{5}$$

Here [B] designates the concentration of the basic form of buffer. The values of $k_4$ given in Table I were determined by linear least-squares fit of the data for each buffer (such as shown in Figure 1, a and b) to (5). The form of (5) demonstrates that the slope of Figure 1b is $1/k_4$. This plot is representative of all of our experiments in showing that the slopes of plots of $1/v$ vs. $1/[B]$ are independent of the concentration of $CO_2$.

Product inhibition by $HCO_3^-$ in (2) and (3) can be understood from the more complete form of the rate equation shown in (6).

$$\frac{v}{[E]_t} = \frac{k_{cat}[B]}{K_m^B\left[1 + \frac{[HCO_3^-]}{K_i^{HCO_3^-}}\left(1 + \frac{K_i^{CO_2}}{[CO_2]}\right)\right] + [B]\left(1 + \frac{K_m^{CO_2}}{[CO_2]}\right)} \tag{6}$$

Here $K_i^{HCO_3^-}$ and $K_i^{CO_2}$ are the inhibition constants for $HCO_3^-$

and $CO_2$. In this equation, $k_{cat}/K_m^B = k_4$. We have measured the inhibition by bicarbonate of the hydration of $CO_2$ at various concentrations of buffers such as Hepes, triethanolamine, 1-methylimidazole, and 4-methylimidazole. Figure 2 is representative of all of our experiments; this double-reciprocal plot strongly suggests linear competitive inhibition with respect to buffer at constant and unsaturating $CO_2$ concentration. Weighted linear least-squares treatment of the data according to a linear competitive inhibition scheme yielded the best fits for all four buffers used. The values of the apparent inhibition constant $K_i^{HCO_3^-}{}_{app}$ obtained in this manner are summarized in Table II. As predicted by (6) and the mechanism of (2) and (3), the values of $K_i^{HCO_3^-}{}_{app}$ we obtain are smaller than the value of 250 mM for $K_{eff}^{HCO_3^-}$, the apparent substrate dissociation constant determined under equilibrium conditions.[14] This is more evident in (7), which

$$K_i^{HCO_3^-}{}_{app} = K_i^{HCO_3^-}/(1 + K_i^{CO_2}/[CO_2]) \quad (7)$$

describes the relationship between $K_i^{HCO_3^-}$ (the true substrate dissociation constant) and the apparent value ($K_i^{HCO_3^-}{}_{app}$) that we have measured based on (6). Equation 7 predicts that only at very high concentrations of $CO_2$ will the apparent and true $HCO_3^-$ inhibition constants be equal. At more modest concentrations of $CO_2$, the apparent value $K_i^{HCO_3^-}{}_{app}$ will be lower than the true value. It is interesting to note that the results of Table II suggest a value for $K_i^{CO_2}$ (the true enzyme–$CO_2$ dissociation constant) of about 0.2 M.

## Discussion

Buffers in solution are able to participate as proton-transfer agents in the mechanism of hydration of carbonic anhydrase II.[2-6] We investigated the nature of this mechanism at concentrations of buffer for which the transfer of a proton from enzyme to external buffer is rate determining. The initial velocity patterns observed are consistent with the proposal of Steiner et al.[15] that the intermolecular proton transfer occurs in a step separate from the interconversion of $CO_2$ and $HCO_3^-$. It should be noted that the initial velocity patterns observed are also by themseleves consistent with two other mechanisms mentioned here, but these are considered unlikely on the basis of other observations. First, (6) is of the same form as that for a classical ping-pong mechanism[16] in which unprotonated buffer is regarded as the second substrate. The distinction between this mechanism and the one shown in (2) and (3) is subtle, involving only the presence of an enzyme–buffer complex in the ping-pong mechanism. However, to be consistent with the classical ping-pong mechanism, a rather strong interaction of buffer and enzyme is required, with dissociation constants of the order of 1 mM, which has not been observed for any of the buffers in Table I. For example, the presence of buffer does not affect the rate of interconversion of $CO_2$ and $HCO_3^-$ at chemical equilibrum, measured by the exchange line broadening of the $^{13}C$ resonance in $H^{13}CO_3^-$,[14] or by $^{18}O$ exchange.[17] Also, the apparent dissociation constant for the enzyme–$HCO_3^-$ complex is independent of buffer concentration.[14] Moreover, there are not large deviations from the Brønsted plot in Figure 3 that would be expected for strong binding of buffer to the enzyme. Second, we also consider the possibility of the formation of a ternary complex between enzyme, $CO_2$, and buffer. Formally, this would result in an ordered bi-bi mechanism, which, under certain conditions, can mimic the initial velocity patterns we have obtained.[16] However, this mechanism is incompatible with the bicarbonate inhibition data reported here unless the true dissociation constant of buffer from the enzyme–$CO_2$ complex is much less than the steady-state $K_m$ value. Since we have already discounted the notion of strong interaction between buffer and enzyme as well as the role of buffer in the interconversion of $CO_2$ and bicarbonate as outlined above, we regard this mechanism as very unlikely.

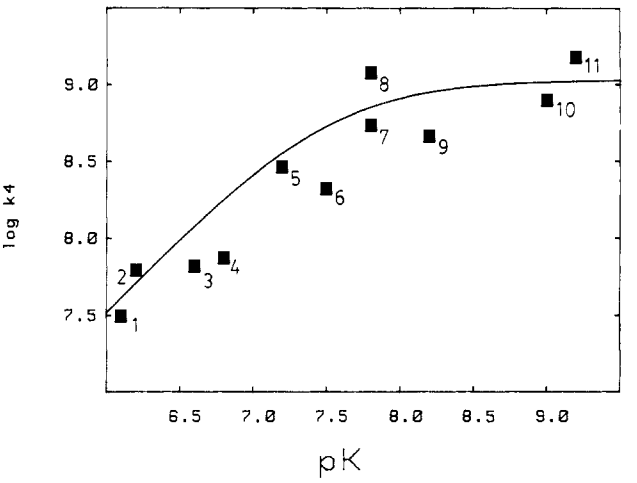


**Figure 3.** Variation of log $k_4$ with p$K_a$ of external buffers: (1) Mes, (2) 3,5-lutidine, (3) 3,4-lutidine, (4) 2,4-lutidine, (5) 1-methylimidazole, (6) Hepes, (7) triethanolamine, (8) 4-methylimidazole, (9) 1,2-dimethylimidazole, (10) Ted, (11) Ches. The curve drawn through the data points was calculated for $k_4 = 1.1 \times 10^9$ $M^{-1}$ $s^{-1}$ and p$K_{enz} = 7.6$ (see text).

Since the available data are not compatible with the existence of an enzyme–buffer complex, we address the question of whether the intermolecular proton transfer has kinetic properties similar to those for proton transfer between small molecules. On the basis of the above arguments, $k_4$ represents the rate constant of proton transfer between enzyme and buffer under the condition that the complex does not sequester a form of the enzyme participating in the interconversion of $CO_2$ and $HCO_3^-$. A plot of log $k_4$ against p$K$ of buffer is shown in Figure 3 and has the appearance expected for a proton-transfer reaction.[18] When the p$K$ of the buffer as proton acceptor is greater than the p$K$ of the enzyme as donor, $k_4$ is independent of the p$K$ value of the buffer in a diffusion-controlled process. When the p$K$ of donor and acceptor are approximately equal, there is a transition, leading to a region in Figure 3 of slope equal to unity when the p$K$ of the buffer is much less than that of the donor on the enzyme. The rate constant for the proton transfer can be approximated by the expression $(k_4)_{max}/(1 + K_{buffer}/K_{enz})$ for the case when the rate constant for the separation from the enzyme of B is equal to that of $BH^+$.[18] $K_{buffer}$ and $K_{enz}$ are the acid dissociation constants for the buffer and for the donor group on the enzyme. The curve in Figure 3 is a best fit of the data to this equation, which yields these least-squares values and standard errors p$K_{enz} = 7.6 \pm 0.6$ and $(k_4)_{max} = (1.1 \pm 0.9) \times 10^9$ $M^{-1}$ $s^{-1}$. The large standard errors could arise from a number of sources. The possibility of the binding of buffer to and inhibition by buffer of the enzyme remains a possibility. We have attempted to minimize such errors by keeping the buffer concentrations below 12 mM but have noticed, for example, that Mes is inhibitory (50 mM Mes at pH 6 causes an inhibition of hydration that is greater than 50%). Another likely source of scatter in Figure 3 is that, in order to use buffers that do not interact greatly with the enzyme, we have selected buffers from several different classes: piperazine and morpholine buffers (Hepes, Mes), secondary and tertiary amines (including Ted and Ches), imidazoles, and pyridines. We have not observed any patterns or trends in values of $k_4$ with changes in structure. Jonsson et al.[5] have presented six values of $k_B$ (equivalent to our $k_4$) that, with some scatter, fall near the curve in Figure 3. The value of $k_4$ for 1,2-dimethylimidazole of Jonsson et al. is smaller by about one-half than the value we have observed. Such a difference might be due to the larger concentrations of buffer used by Jonsson et al.[5] or to the different pH at which observations

(14) Simonsson, I.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1979**, *93*, 409–417. Steiner, H.; Jonsson, B. H.; Lindskog, S. *FEBS Lett.* **1976**, *62*, 16–20.
(15) Steiner, H.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1975**, *59*, 253–259.
(16) Segel, I. H. "Enzyme Kinetics"; Wiley: New York, 1975; pp 505–656.
(17) Silverman, D. N.; Tu, C. K.; Lindskog, S.; Wynns, G. C. *J. Am. Chem. Soc.* **1979**, *101*, 6734–6740.
(18) Eigen, M. *Angew. Chem., Int. Ed. Engl.* **1964**, *3*, 1–19.

were made (Jonsson et al. measured at pH 8.9; we measured at 8.5).

The value of $pK_{enz} = 7.6 \pm 0.6$ that we have estimated is consistent with the possibility that His-64 in the active-site cleft, which has been determined by NMR to have a $pK_a$ near 7,[19] is the proton acceptor on the enzyme. In this case, His-64 could act as a proton shuttle to the activity-controlling group itself, for which there is evidence from isotope effect studies.[15,20] The data of Figure 3 considered alone are also consistent with proton transfer directly between the buffers and the activity-controlling group of $pK_a$ near 7.

The data in Figure 3 appear to be in a transition region of the Brønsted plot, with values of $k_4$ at pH near 9 approaching a plateau. The estimated limiting value of $k_4$ from Figure 3 of $(1.1 \pm 0.9) \times 10^9$ $M^{-1}$ $s^{-1}$ can be compared with proton-transfer reactions between small molecules for which imidazole acts as a proton acceptor. In such a case, the diffusion-controlled value is approximated by the rate constant of $1.2 \times 10^9$ $M^{-1}$ $s^{-1}$ for proton transfer from acetic acid ($pK_a = 4.8$) to imidazole.[21] In the reverse direction, the diffusion-controlled value is approximated by the rate constant of $2 \times 10^{10}$ $M^{-1}$ $s^{-1}$ for proton transfer from imidazole to carbonate ($pK_a = 10.3$).[21] This interesting comparison demonstrates that the diffusion-controlled limit in a proton-transfer reaction involving buffer and the enzyme carbonic anhydrase is at least within a factor of 20 of the value for a small-molecule counterpart. This result is not likely to be influenced by limitations of the changing pH indicator method; the rate constant for combination of, for example, the indicator *p*-nitrophenol with $H^+$ is in excess of $10^{10}$ $M^{-1}$ $s^{-1}$.[22]

It is our conclusion that, at least for low concentrations (<12 mM) of buffers, the rate-limiting proton transfer between human carbonic anhydrase II and external buffer is dependent on the $pK$ difference between donor and acceptor species in a manner consistent with proton transfer between small molecules. The rate constant for the proton transfer follows a Brønsted curve that reaches a plateau at a value, $1 \times 10^9$ $M^{-1}$ $s^{-1}$, very close to that found for proton transfer between small molecules. The transition region of the Brønsted plot indicates that the donor group on the enzyme has a $pK_a = 7.6 \pm 0.6$, which is consistent with proton transfer directly between the active site and buffer or between a proton shuttle group on the enzyme of $pK_a$ near 7 and buffer.

**Acknowledgment.** We thank Professor Sven Lindskog and Dr. K. S. Venkatasubban for their very helpful comments. This work was supported by a grant from the National Institutes of Health (GM 25154).

**Registry No.** $CO_2$, 124-38-9; Mes, 4432-31-9; Hepes, 7365-45-9; Ted, 280-57-9; Ches, 103-47-9; 3,5-lutidine, 591-22-0; 3,4-lutidine, 583-58-4; 2,4-lutidine, 108-47-4; 1-methylimidazole, 616-47-7; triethanolamine, 102-71-6; 4-methylimidazole, 822-36-6; 1,2-dimethylimidazole, 1739-84-0; carbonic anhydrase II, 9001-03-0.

(19) Campbell, I. D.; Lindskog, S.; White, A. I. *J. Mol. Biol.* **1975**, *98*, 597-614.
(20) Venkatasubban, K. S.; Silverman, D. N. *Biochemistry* **1980**, *19*, 4984-4989.
(21) Eigen, M.; Hammes, G. G. *Adv. Enzymol. Relat. Subj. Biochem.* **1964**, *25*, 1-38.
(22) Eigen, M.; Kustin, K. *J. Am. Chem. Soc.* **1960**, *82*, 5952-5953.

# Interactions between Bilirubin and Albumins Using Picosecond Fluorescence and Circularly Polarized Luminescence Spectroscopy

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**Abstract:** Circular dichroism, circularly polarized luminescence, and time-resolved picosecond fluorescence spectroscopy have been used to study the interaction between bilirubin (BR), human serum albumin (HSA), bovine serum albumin (BSA) at several pH's, albumin/BR ratios, and salt concentrations. The BR single excited state when bound to alubmin is in a right-handed helical conformation, similar to that of the ground state except near pH 4.1, where the ground state takes up a left-handed conformation. Solutions of bilirubin exhibit biexponential fluorescence decays, with the major decay (~30 ps) being attributable to BR while the minor decay (~1 ns) is due to photoproducts of BR, possibly *Z,E*, *E,Z*, and *E,E* geometric isomers. The maximum rate of photoisomerization from BR is calculated as $5 \times 10^{10}$ $s^{-1}$ and that of the isomers is $5 \times 10^8$ to $10^9$ $s^{-1}$. When bound to HSA, bilirubin and its photoproduct appear to attach themselves to different types of binding sites since BR excited singlet decay times are unaffected by salt or pH whereas the photoproducts decay times are sensitive to these factors.

About 20% of newborn infants develop jaundice during their first 3-4 days because of the inefficiency of their hepatic function and the short lifetime of their red blood cells.[1] Bilirubin (BR),[2] the catabolic product of blood heme, builds up in the blood plasma, enters fatty tissue, and hence causes neonatal jaundice.[1] Bilirubin binds strongly to serum albumin, and this binding lowers the level of free BR in blood plasma.[3] Because of the small amount of albumin in newborn infants, it is necessary to perform phototherapy in order to lower BR levels of those suffering from extreme jaundice, thus preventing the damaging effects of BR on the brain (kernicterus) and other organs.[1,4]

The binding of bilirubin to albumins has been studied extensively by NMR,[5] visible absorption,[6] CD,[6,7] ORD,[8] and fluorescence

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(1) (a) Karp, W. B. *Pediatrics* **1979**, *64*, 361-368. (b) Brodersen, R. *CRC Crit. Rev. Clin. Lab. Sci.* **1980**, *11*, 305-399.
(2) Abbreviations: BR, (4*Z*,15*Z*) bilirubin IX$\alpha$, PBR, photobilirubin or *E,Z*, *Z,E*, or *E,E* isomers of BR; HSA, human serum albumin; BSA, bovine serum albumin; HBR, 3,4-diethyl-5-(3,4-diethyl-5-methylpyrrol-2-yl methilidene)-3-pyrolin-2-one.
(3) Chen, R. F. *Arch. Biochem. Biophys.* **1974**, *160*, 106-112.
(4) McDonagh, A. F.; Palma, L. A.; Lightner, D. A. *Science (Washington, D.C.)* **1980**, *208*, 145-151.

0002-7863/82/1504-6741$01.25/0 